UNIVERSITI PUTRA MALAYSIA

MOLECULAR CLONING AND FUNCTIONAL STUDIES OF SILICON-RESPONSIVE SERINE-RICH PROTEIN TRANSCRIPTS FROM MANGROVE PLANT, *Rhizophora apiculata* (Blume)

MAHBOD SAHEBI

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By

MAHBOD SAHEBI

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

February 2014

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DEDICATED TO

My Father Mansoor Sahebi;
My Mother Mehri Sahebi;
My beloved wife Parisa Azizi;
My Brother and sister

Abstract of thesis submitted to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for Doctor of Philosophy

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Chairman : Professor Mohamed Hanafi Musa, PhD
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Silicon (Si) is one of the most plentiful elements found in the soil. Silicon plays an important role in decreasing susceptibility of plants against variety of different biotic and abiotic stresses. Mangrove plant (*Rhizophora apiculata*) is able to accumulate, and process Si to generate biosilica. Therefore, it would be a beneficial source for genetic manipulation of susceptible plants in the stress conditions. The objectives of the study were (i) to identify and characterize of a Si responsive gene in mangrove, (ii) to analyze the expression levels of a gene encoding *serine-rich protein*, and (iii) Functional studies of *serine-rich protein* in *Arabidopsis thaliana.* Three different methods and RNeasy plant mini kit were used to extract nucleic acids. The Suppression Subtractive Hybridization (SSH) technique was used to remove transcripts from proteins which were not involved in Si accumulation. Specific primer was designed to get full-length CDS of *serine-rich protein.* Semi-quantitative RT-PCR and real-time PCR were performed to examine its expression level under the control and treatment conditions. The Gateway Technology was used to construct entry and the expression vectors. Transformation of *Arabidopsis thaliana* with *serine-rich protein* gene was performed using *Agrobacterium*-mediated transformation by the floral-dip method. Energy-dispersive X-ray spectroscopy and high performance liquid chromatography were used to measure the quantity of Si and serine amino acid, respectively. Modified CTAB and SDS were quick and reliable methods for isolation of total RNA from the roots and leaves of mangrove, respectively. Of the sequences obtained from cDNA library, four were 97% similar to *serine-rich protein* gene of groundnut (*Arachis hypogaea*). Full-length of the *serine-rich protein* cDNA obtained through amplification of the cDNA template using specific primers. The

expression levels of *serine-rich protein* transcript were generally higher in the Si treated mangrove plants than untreated plants. The amount of serine amino acid of transgenic *Arabidopsis* has increased significantly from 1.02 mg g^{-1} in wild-type plants to 37.76 mg g^{-1}. In addition, concentration of Si in the leaves and roots of transgenic plant was significantly higher than that in the wild type ($P<0.01$). This study successfully determined the Si responsive transcript related to *serine-rich protein* in mangrove plant (*R. apiculata*).

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Doktor Falsafah

PENGKLONAN MOLEKUL DAN PENCIRIAN FUNGSI *TRANSKRIP PROTEIN SILIKON*-RESPONSIF SERINE-KAYA DARI PADA TUMBUHAN BAKAU, *Rhizophora apiculata* (Blume)

Oleh

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ABSTRAK

Pengerusi : Professor Mohamed Hanafi Musa, PhD
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Silikon (Si) adalah salah satu elemen yang paling banyak didapati dalam tanah. Silikon memainkan peranan yang penting dalam mengurangkan kerentanan tumbuhan terhadap pelbagai tekanan biotik dan abiotik. Tumbuhan bakau (*Rhizophora apiculata*) mampu mengumpul dan memproses Si untuk menjana biosilika. Oleh itu, ia berupaya menjadi sumber bermanfaat untuk memanipulasikan genetik tumbuhan yang terdedah kepada tekanan persekitaran. Objektif kajian ini adalah (i) untuk mengenalpasti dan mencirikan gen Si-responsif dalam tumbuhan bakau, (ii) untuk mengesan tahap pengekespresan gen yang mengekod protein serine-kaya, dan (iii) kajian fungsi *protein serine-kaya* dalam *Arabidopsis thaliana*. Tiga kaedah yang berbeza dan RNeasy plant mini kit telah digunakan untuk mengeluarkan asid nukleik. Teknik Suppression Subtractive Hybridization (SSH) telah digunakan untuk mengasingkan protein transkrip yang tidak terlibat dalam pengumpulan Si. Buku asas khusus yang telah direka untuk mendapatkan CDS penuh panjang protein serine yang kaya. Separuh-kuantitatif RT-PCR dan tepat masa PCR telah dijalankan untuk mengkaji tahap ungkapan di bawah kawalan dan rawatan syarat-syarat. Gateway teknologi telah digunakan untuk fungsi pembinaan vektor. Transformasi *Arabidopsis thaliana* dengan *protein serine-kaya* gen telah dilakukan melalui *Agrobacterium* dengan keadah pencelupan bunga. Tenaga-serakan X-ray spektroskopi dan kromatografi cecair prestasi tinggi telah digunakan untuk mengukur kualiti Si dan asid amino serine. CTAB dan SDS yang diubahsuai merupakan dua kaedah yang cepat dan sesuai untuk pengeluaran asid nukleik RNA daripada akar dan daun bakau. Empat urutan yang diperolehi daripada jumlah perpustakaan cDNA menunjukkan 97% persamaan dengan jujukan penuh *protein serine-kaya* gen dari kacang tanah (*Arachis hypogaea*). Jujukan penuh *protein serine-kaya* diperolehi melalui amplifikasi template cDNA

menggunakan primer tertentu. Hasil kajian menunjukkan bahawa tahap pengekespresan serine-kaya protein transkrip adalah lebih tinggi dalam tumbuhan bakau yang dirawat dengan Si berbanding dengan yang tidak dirawat. Jumlah asid amino serine dalam transgenik *Arabidopsis* telah meningkat dengan ketara dari 1.02 mg g^{-1} dalam tumbuh-tumbuhan jenis liar sehingga 37.76 mg g^{-1}. Di samping itu, kandungan Si dalam daun dan akar tumbuhan transgenik adalah jauh lebih tinggi daripada jenis liar *(*$P < 0.01$). Kajian ini berjaya menentukan Si-responsif transkrip adalah berkaitan dengan *protein serine-kaya* dalam tumbuhan bakau (*R. apiculata*).

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I certify that a Thesis Examination Committee has met on 13 February 2014 to conduct the final examination of Mahbod Sahebi on his thesis entitled "Molecular Cloning and Functional Studies of Silicon-Responsive Serine-Rich Protein Transcripts from Mangrove Plant, *Rhizophora apiculata* (Blume)" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

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LIST OF ABBREVIATIONS

A. *tumefaciens*	*Agrobacterium. tumefaciens*
Asp	Aspartic acid
Arg	Arginine
Ala	Alanine
aa	Amino acid
Bp	Base Pairs
cDNA	Complementary DNA
CTAB	Hexacetyltrimethyl ammonium bromide
DEPC	Diethyl pyrocarbonate
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
$dNTP_S$	deoxynucleotides
Ds	Double-stranded
EDTA	Ethylene diamine tetra acetic acid
EDX	Energy-dispersive X-ray spectroscopy
EtBr	Ethidium bromide
G	Gram
Glu	Glutamic acid
Gly	Glycine
His	Histidine
Hr	Hour
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
Ile	Isoleucine
kb	Kilo base-pair
L	Liter
LB	Luria-bertani
LiCl	Lithium chloride
Lys	Lysine
Leu	Leucine
M	Molar
min	Minure
Met	Methionine
mg	Milligram
mg g^{-1}	Miligram per gram
mL	Milliliter
mM	Millimolar
mRNA	Massenger RNA
NaCl	Sodium chloride
NCBI	National Center for Biotechnology Information
ng	Nanogram
OD	Optical density
ORF	Open reading frame

OH	Hydroxide
PCR	Polymerase chain reactions
PVP	Polyvinylpyrrolidone
Pro	Proline
Phe	Phenylalanine
RNA	Ribonucleic acid
RT	Room Tempreture
RT-PCR	Reverse transcriptase polymerase chain reaction
RNase	Ribonuclease
g (rcf)	Gravity
ROS	Reactive oxygen species
SSH	Suppression Subtractive Hybridization
SDS	Sodium dodecyl sulphate
Sec	Second
SEM	Scanning electron microscope
Ser	Serine
Si	Silicon
ss	Single-stranded
spp	Species
TAE	Tris acetate EDTA
TBE	Tris borate EDTA
TE	Tris-EDTA
T-DNA	Transfer DNA
Thr	Threonine
Tyr	Tyrosine
Val	Valine
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
$\mu g\ \mu L^{-1}$	Microgram per microlitre
µL	Microliter
µg	Microgram
˚C	Degree Centigrade
%	Percentage

CHAPTER 1

INTRODUCTION

1.1 General Introduction

One of the highly prevalent elements among the soil ingredients is silicon (Si). Silicon is considered as a non-essential nutrient element for a large number of plant species. Absorption of Si by plants may protect them against a variety of different abiotic stresses including heavy metal toxicity (Neumann and Zur Nieden, 2001), salinity (Tahir *et al*., 2006), drought (Lux *et al*., 2002), disproportion of soil nutrients (Jianfeng and Takahashi, 1990; Ma, 2004) and climate changes (Agarie *et al*., 1998; Ma *et al*., 2001b). Besides, absorption of Si may increase tolerance of plants to some biotic stresses such as pathogens and pests (Ishiguro, 2001; Meyer and Keeping, 2001). Hence, Si can have an effect on both the yield and quality of agricultural crops.

Several processes such as addition, transformation, deduction, and translocation of different particles are involved in soil formation. Silicate minerals are the most important factors in chemical transformation inside the soil. Minerals rich in Si differ in the intensity and period of numerous specific processes involved in soil formation (Korndörfer and Lepsch, 2001). Role of Si as a fertilizer has been reported widely by both horticulturists and agronomists in certain soils leading to increased crop yield and quality (Epstein, 2001). Silicon is the eighth common element in the world which is found in compounds, and it hardly ever occurs as an untainted free element in the nature. The most abundant element in the soil after the oxygen is Si appears in two forms silica and oxides of silicon. The Si is dispersed widely as different forms of silica in the sands, plants, dusts, and planetoids. Silicate minerals form the major portion of the Earth's crust (Ma, 2004). Silicon is considered as a useful element for plant formation and growth, and its absorption by plants helps them to overcome different abiotic and biotic stresses (Ma, 2004). Silicon is used in different industrial fields, such as industrial building in tainted form and extracted through a few steps of processing of the natural compounds to make ceramic brick, concrete. Pure Si is also used in aluminum-casting, making fumed silica, and steel refining; extremely purified Si is used in semiconductor electronics. In biology field tiny trace of Si acts as a vital element for animals (Nielsen, 1984). A variety of microorganisms, such as diatoms use Si to form their structures. Besides to all above-mentioned roles of Si, it has an important role in plants especially in grass metabolism.

Biogenic silica is made by diatoms in the environment, however, it seems that there is a lack in structural control of the process. Moreover, producing of inorganic silica at ambient conditions needs extreme temperatures and pHs (Iler, 1979). These limitations of producing silica in the environment encouraged material scientists to synthesis biomimetic silica and use it in industrial as well as electronic devices (Morse, 1999; Tacke, 1999; Vrieling *et al*., 1999). Use of organic molecules has been suggested in the silica biomineralization process (Kinrade *et al*., 1999; Zhou *et al*., 1999; Perry and Keeling-Tucker, 2000; Sahai and Tossell, 2001), however, complexes of organic Si have been assumed to be effective on Si uptake and transportation (Hildebrand *et al*., 1997; Da Silva and Williams, 2001; Sahai and Tossell, 2001). In the current study, Si has been selected due to its important function in crops and sustainable agricultural systems. The molecular mechanisms of Si uptake in most plant species, except for some cereal such as rice and maize, are poorly examined. Silicon absorption and transportation mechanism most probably is genetically different between plants even between different species of the same genus. For instance, three different genes have been identified as Si uptake and transporter genes in the roots and leaves of rice (Ma *et al*., 2008), while their role and localization are different in maize (Mitani *et al*., 2009).

Mangrove as woody plants, growing in tropical and sub tropical areas, have a high range of adaptability to different harsh environmental situations and pathogens as well. Because of their particular habitat, mangrove plants may be providing a valuable source of genes responsible for tolerance to a wide range of biotic and abiotic stresses. It has been reported that mangrove plants are able to absorb large amount of Si from the soil through their specific roots and transfer to shoot parts. Therefore, this hypotheses is derived that mangrove would be a beneficial source for genetic manipulation of susceptible plants exposed to stress condition to absorb and accumulate more silicon. Mangrove plants are able to successfully grow in intertidal areas, where sediments usually formed under shortage of essential plant nutrients, oxygen, and accretion of soluble phytotoxins, such as H_2S, CH_4, Fe^{2+} and Mn^{2+}(Ponnamperuma, 1984). This is attributed to their anatomical adaptation leading to transport sufficient amount of oxygen to below-ground parts of the roots (Koncalová, 1990; Kludze *et al*., 1993; Youssef and Saenger, 1996; Cheng *et al*., 2010).

Mangrove plants are able to accumulate, store and process Si to generate biosilica, which theoretically supposed to be similar to what happens in sponges and diatoms. To understand *in vivo* silica formation and study the required environment for biosilica occurrence, one approach is the isolation and characterization of different sequences of amino-acids and

their structure, association to their related protein functions, followed by investigation of the role of proteins in silica formation *in vitro* (Kauss *et al*., 2003).

This study investigated the gene responsible for Si accumulation and transportation in mangrove seedling after treatments with Si, using suppression subtractive hybridization (SSH) as a molecular biology technique to make a subtractive cDNA library and examine the expression levels of Si responsive gene. The use of SSH technique lead to remove proteins with the same regulatory functions than those involved in Si uptake and accumulation. There is no information available on the literature relating to the mechanisms in association with Si uptake by roots of mangrove plants. The mechanism of Si accumulation and transportation in mangrove plants should be different with other higher plants and may be controled by different genes.

The organic substances related to biogenic silica synthesis involves glycoproteins and polysaccharides enriched in hydroxyl terminated group amino acids containing serine, glycine, threonine, glutamic acid and aspartic acid (Hecky *et al*., 1973; Swift and Wheeler, 1992; Vrieling *et al*., 1999). Although it is assumed that biosilica can be produced by organisms, exact details of relevant procedures have not been discovered yet. Organic environment includes carbohydrates, lipids, proteins, metal ions and phenolic components in plants, which probably play an elementary role in producing biosilica (Perry and Lu, 1992; Harrison, 1996). Several studies have been done to examine the effect of different amino-acids including (glutamine, serine, lysine, proline, threonine, aspartic acid, asparagines, and arginine) and their homopeptides on silica formation (Coradin and Livage, 2001; Belton *et al*., 2004).

This is assumed that the *serine- rich protein* may be involved in Si uptake, transport and silica nucleation in matrix-mediated as a stable intermediate. Theses complexes supposed to involve C-O-Si covalent bonds or H- bonds with Si in different coordination forms (Quadra or Penta) (Swift and Wheeler, 1992; Lobel *et al*., 1996; Kinrade *et al*., 1999; Da Silva and Williams, 2001).

The primary objective of this thesis was to isolate and characterize novel silicon-responsive genes in mangrove (*Rhizophora apiculata*) roots. Further objective was to investigate the role of protein rich in serine with respect to the gene expression regulation in order to decide if serine-rich protein mediates changes in gene expression. The specific objectives of this study were:

1. To analyze silicon induced changes of gene expression in root and leaves of mangrove.

2. To generate Expressed Sequence Tag (EST) library from mangrove after Si treatment.
3. To obtain full-length cDNAs clones corresponding to the identified silicon responsive complete cDNA.
4. To determine the role of protein rich in serine in accumulation of silicon in roots of transformed *Arabidopsis thaliana.*

This study not only grants genetically information involved in Si absorption by roots of mangrove, it also provides significant information resulted in inducing *serine-rich protein* gene in *Arabidopsis thaliana*. Although so far the molecular role of *serine-rich protein* has not been reported in plants, the role of *serine-rich protein* has been widely studied biochemically and provided highlights information on silica formation.

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APPENDICES

APPENDIX A
Formulation for media and solution

1. Composition of hydroponic culture (modified Johnson solution)

Macro elements		**Micro elements** (1mL L^{-1})	
KNO_3	6 mL L^{-1}	KCl	0.932 gr
$Ca(NO_3)_2.4H_20$	4 mL L^{-1}	H_3BO_3	0.386 gr
$NH_4H_2PO_4$	2 mL L^{-1}	$MnSO_4.H_2O$	0.084 gr
$MgSO_4.7H_2O$	2 mL L^{-1}	$ZnSO_4.7H_2O$	0.143 gr
		$CuSO_4.5H_2O$	0.031 gr
		$(NH_4)_6\,Mo_7O_{24}.4H_20$	0.020 gr
		Fe-EDTA	1.0769 gr/50 mL

2. Media for Bacterial growth

LB Agar (1L)		**LB Broth (1L)**	
Agar	15 g	Tryptone	10 g
Tryptone	10 g	NaCl	10 g
NaCl	10 g	Yeast extract	5 g
Yeast extract	5 g		

Add distilled water to final volume of 1L; adjust the pH to 7.0, and autoclave.

Low salt LB (1L)

Tryptone	10 g
NaCl	5 g
Yeast extract	5 g

Add distilled water to final volume of 1L; adjust the pH to 7.0, and autoclave.

3. **Antibiotics**

Ampicillin (100 mg mL^{-1}). Dissolved 1000 mg in 10 ml of sterile water, filter sterilized, and stored at -20˚C.

Kanamycin (50 $mgmL^{-1}$). Dissolved 500 mg in 10 ml of sterile water, filter sterilized, and stored at -20˚C.

Spectinomycin(100 mg mL^{-1}). Dissolved 100 mg in 1 ml of sterile water, filter sterilized, and stored at -20˚C.

Rifampicin. 50 mg mL^{-1}, dissolved in DMSO, added sterile water up to 1 mL, filter sterilized, and stored at -20˚C.

Zeocin (50mg/mL). Dissolved 500 mg in 10 ml of sterile water, filter sterilized, and stored at -20˚C.

4. **Other solutions**

TE buffer

Tris-Hcl 10 Mm
EDTA 1 Mm (pH 8.0)

TBE buffer (1X)

Tris base 10.8 g
Boric acid 5.5 g
EDTA 0.37 g
Add sterile water to a final volume of 1 L.

TAE buffer (1X)

Tris base 4.84 g
Glacial acetic acid 1.142 mL
$Na_2EDTA.2H_2O$ 0.74 g
Add sterile water to a final volume of 1 L.

PCI,CI

Phenol: chloroform: isoamyl alcohol (25: 24: 1)
Chloroform: isoamyl alcohol (24: 1)

Sodium acetate 3M (100mL). Dissolved 40.8 g sodium acetate in sterile water and bring the volume to 100 mL.

APPENDIX B
Figures of vectors map

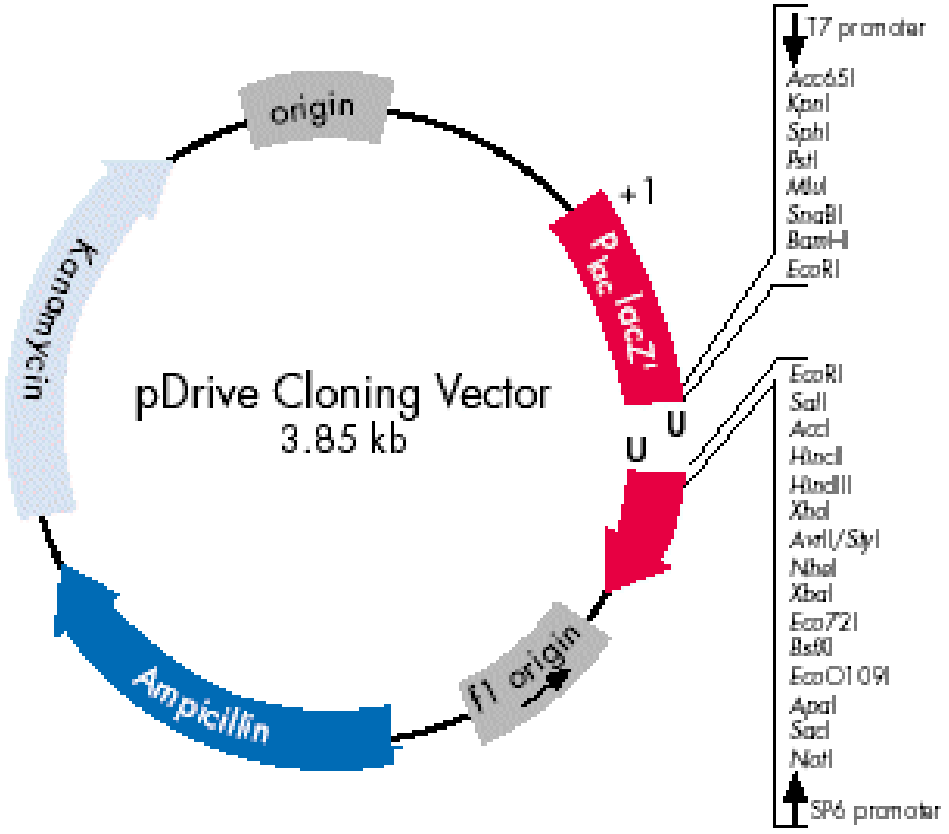


Figure B.1 Plasmid Map of pDrive cloning vector (Qiagen, Germany).

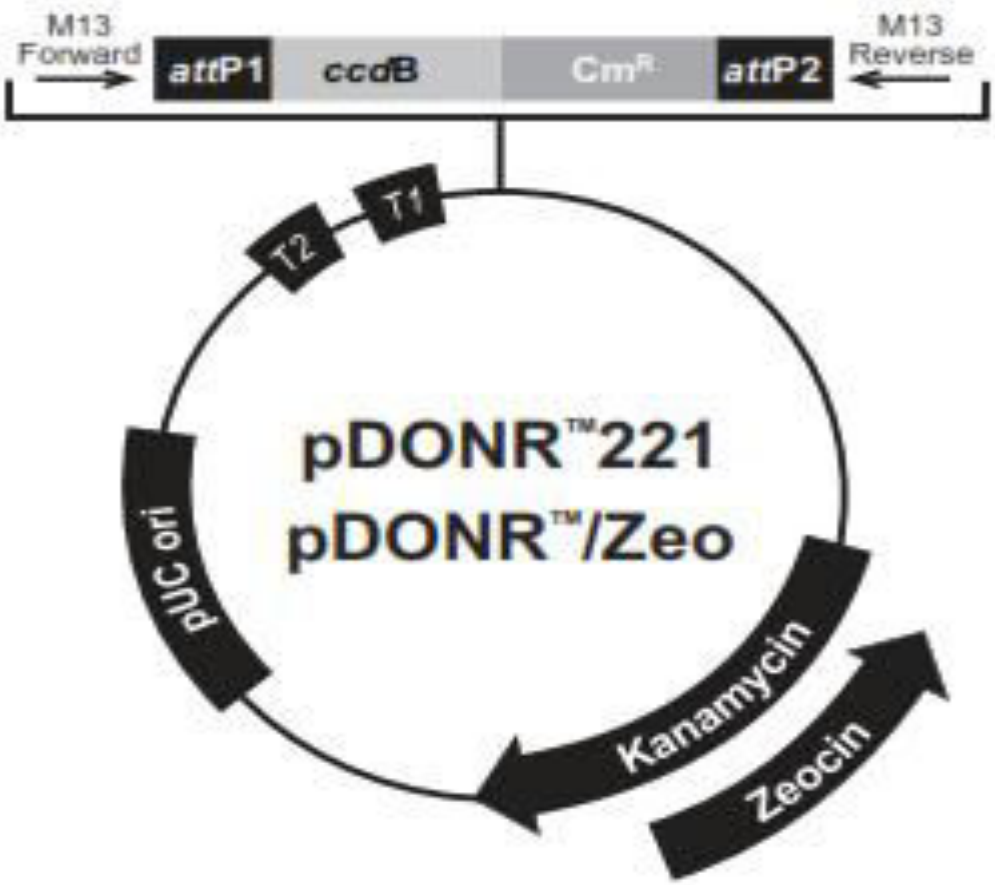


Figure B.2 Plasmid map of pDONOR/Zeo vector (Invitrogen, CA, USA).

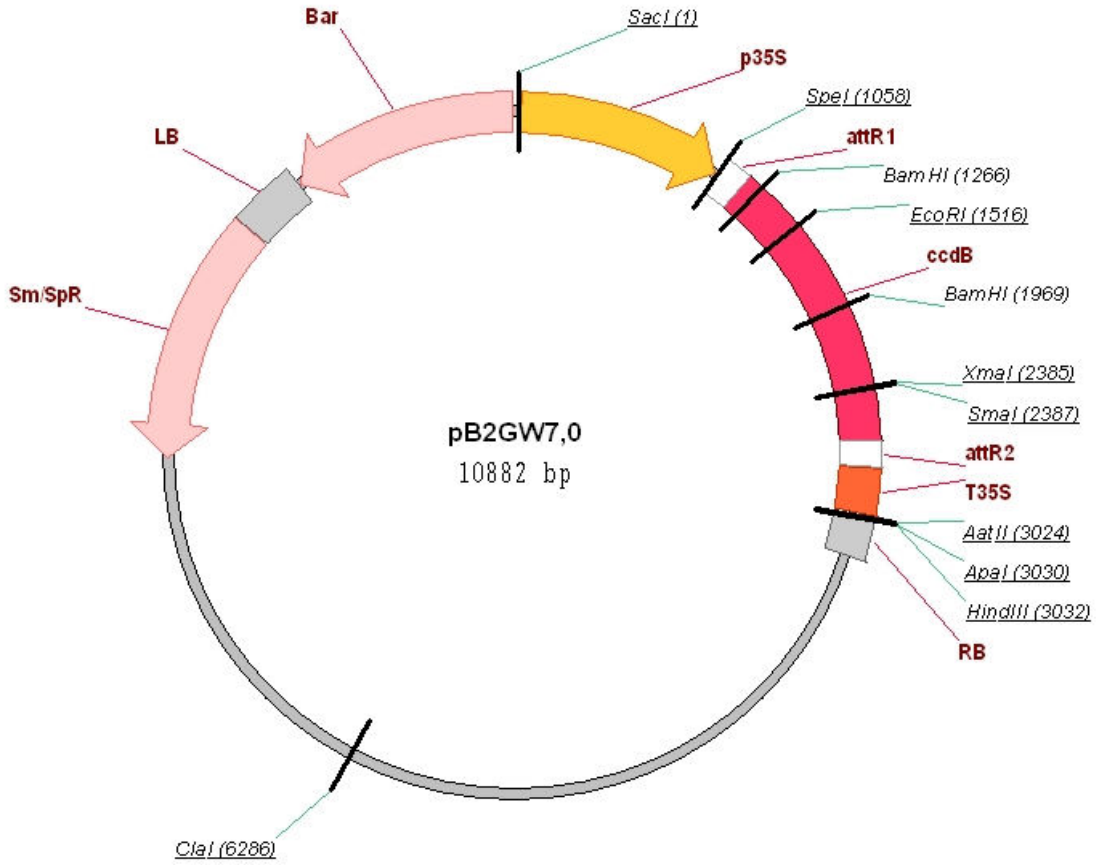
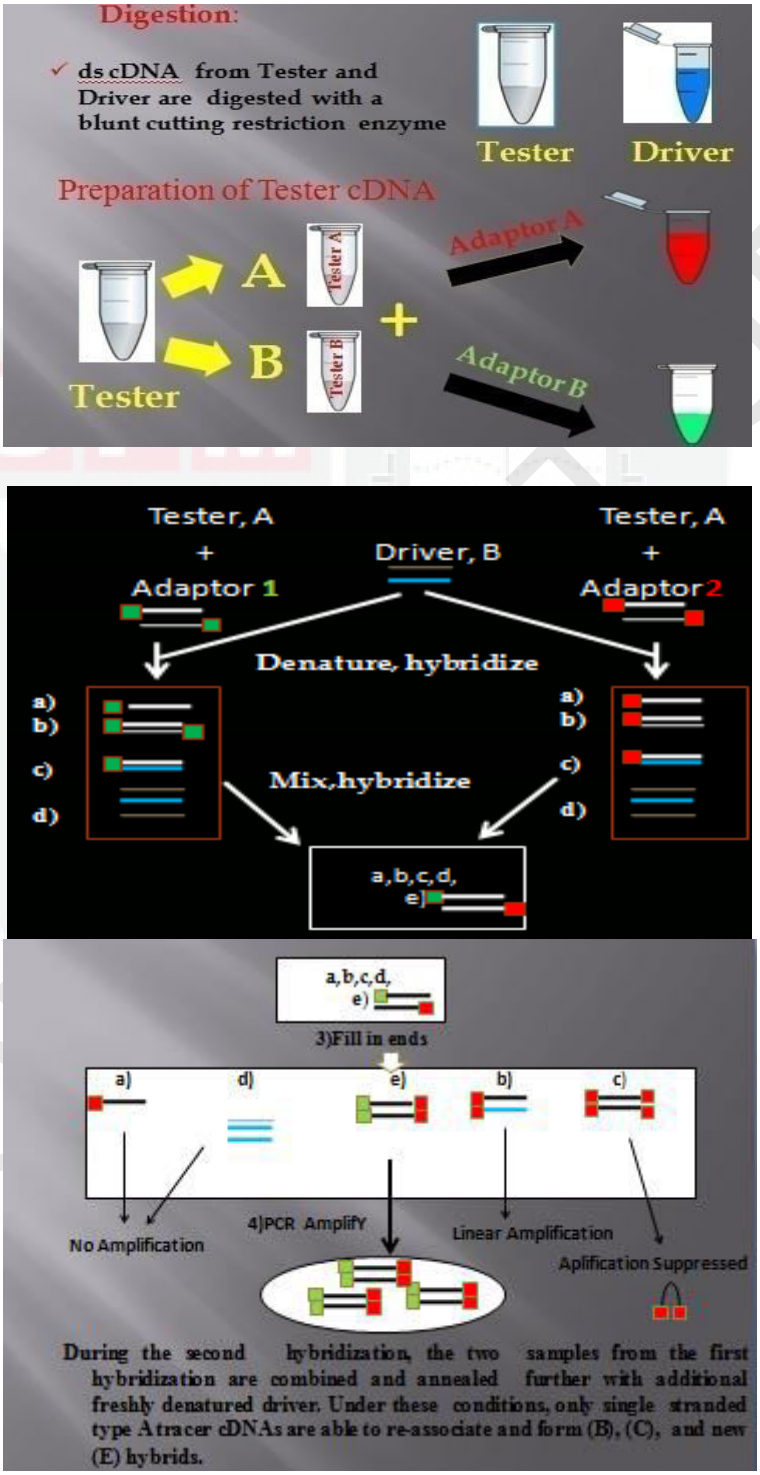


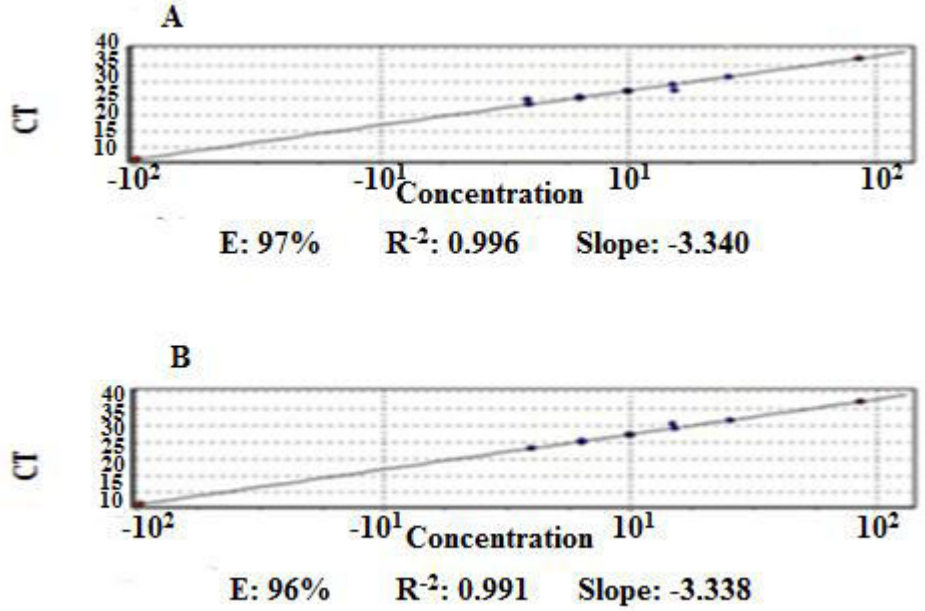
Figure B.3 Plasmid map of pB2GW7vector (Karimi *et al.*, 2002).

APPENDIX C
Schematic mechanism of SSH



APPENDIX D

The standard curve from purified cDNA fragment of internal (A) and *serine rich protein* genes (B).

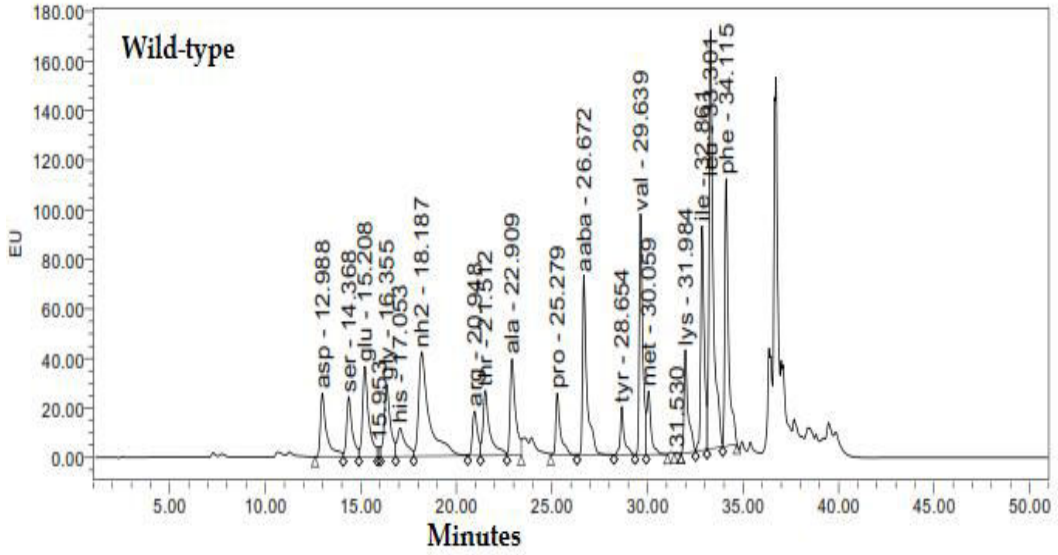


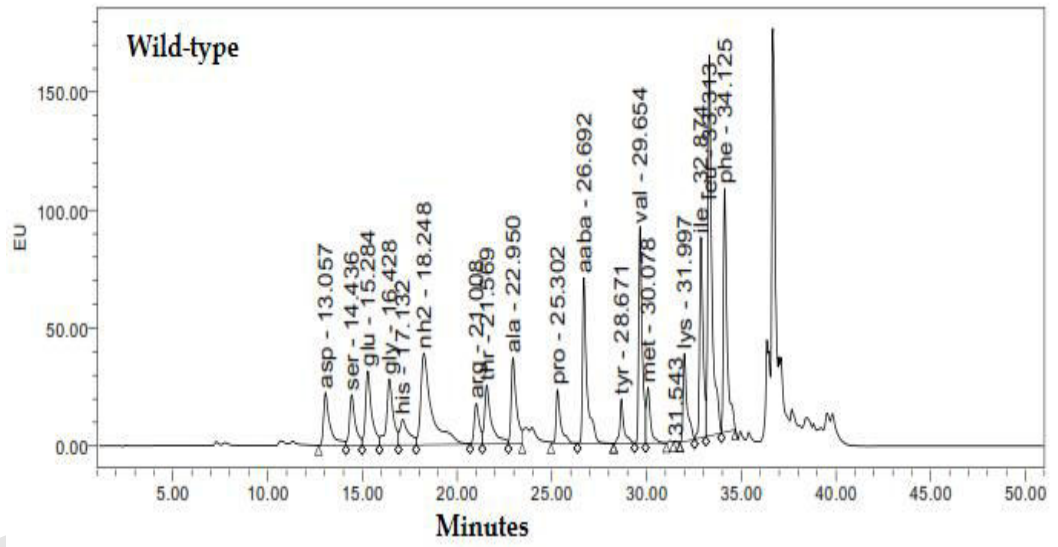
APPENDIX E
Hydroponic culture of mangrove seeds

Image scale must be multiplied by 10 to show the real size image.

APPENDIX F

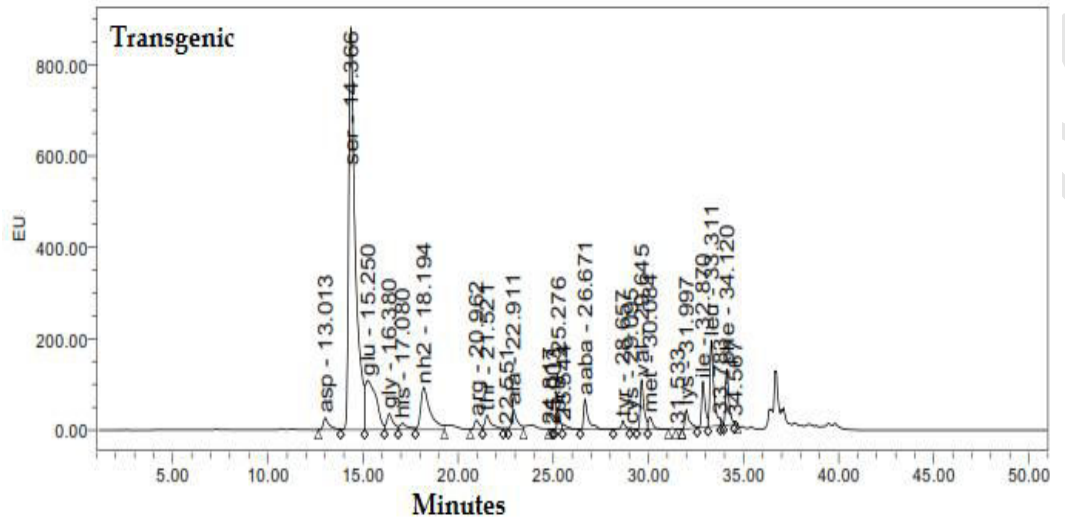
HPLC chromatogerams of amino acids (Replication 2 and 3 of wild-type plants).

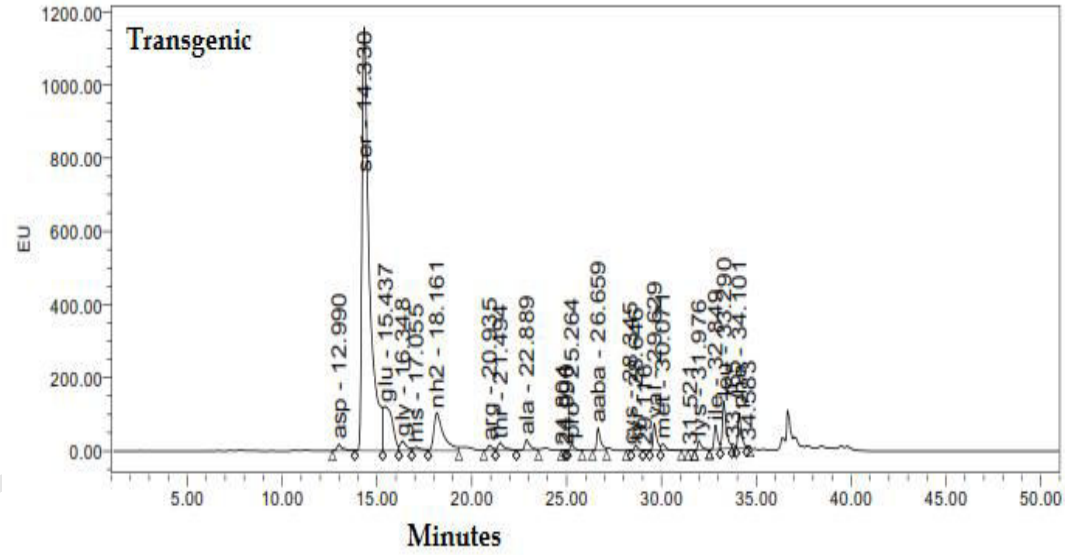




APPENDIX G

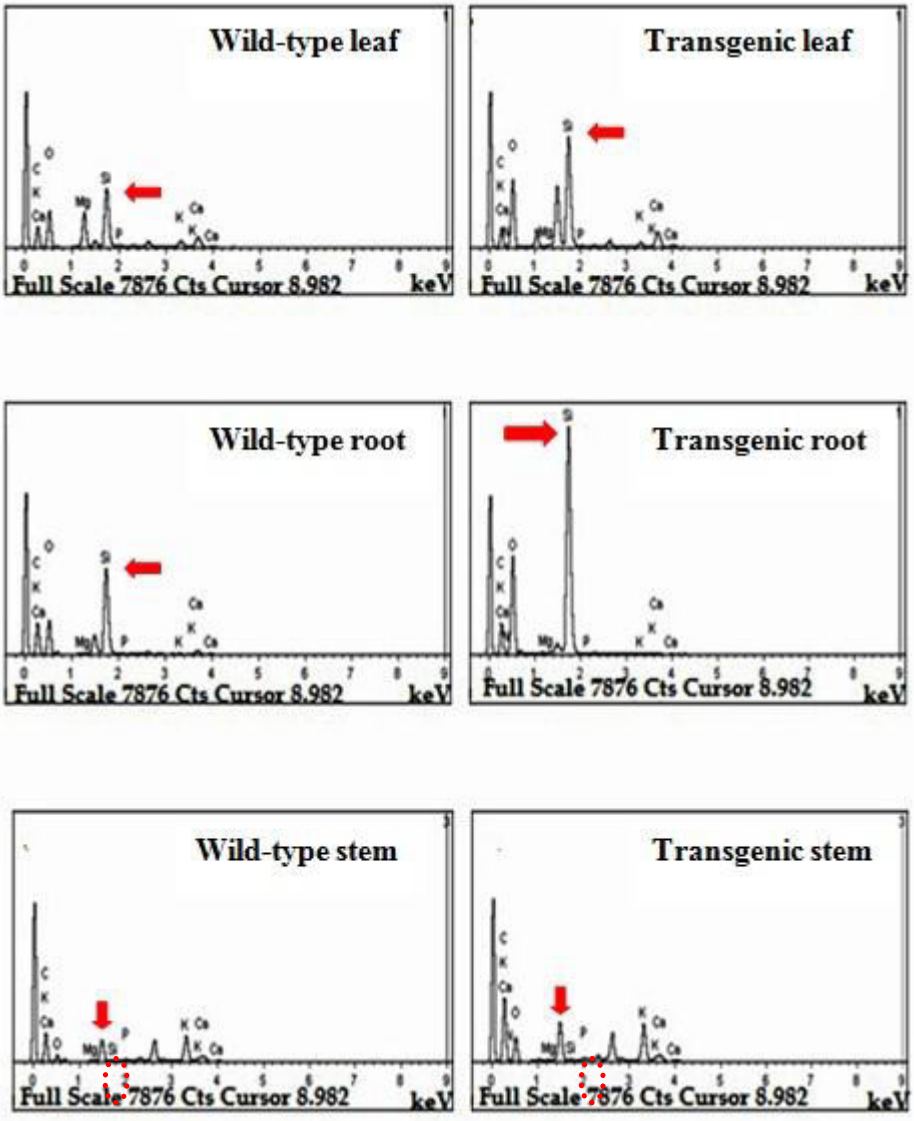
HPLC chromatogerams of amino acids (Replication 2 and 3 of transgenic plant)





APPENDIX H

Atomic percentage of the elements detected from the X-ray point analysis at the specific location for Si treatment.



BIODATA OF STUDENT

Mahbod Sahebi was born on 6 of April 1978 in Ahwaz. He graduated in 2001 with Bachelor of Science in plant breeding from Islamic Azad University, Khorramabad Division and obtained his Master of science in the field of agricultural engineering, branch of agronomy and plant breeding from Islamic Azad University, Borujerd Division with the average of 18.70; He was the top student in MSc. The subject of research in master level was: Cytogenetic and morphologic determination *Rosa damacena mil* with the defense score of 19.80 under supervision of Dr.Ali Reza Tabai Aghdaii. He has worked with Associate Prof Dr.Rajabi Memari for 2 years in Chamran University since 2008. On January 2011, He started his PhD program on plant biotechnology under supervision of Prof. Dr. Mohammed Hanafi Musa in the laboratory of plant crops, Institute of Tropical Agriculture (ITA), Universiti Putra Malaysia. In his post graduate life, he got many opportunities to participate in the several workshops and training programs that were certainly valuable to his research held in Malaysia. He had submitted four papers in the international journals.

LIST OF PUBLICATIONS

Mahbod Sahebi, Mohamed M Hanafi, Siti Nor Akmal Abdullah, Naghmeh Nejat, Mohd Y. Rafii, Parisa Azizi. Extraction of total RNA from mangrove plants to identify different genes involved in its adaptability to the variety of stresses Pak. J. Agri. Sci., Vol. 50(4), 1-9; 2013

Mahbod Sahebi, Mohamed M Hanafi, Siti Nor Akmal Abdullah, Mohd Y. Rafii, Parisa Azizi, Naghmeh Nejat, Abu Seman Idris. Isolation and expression analysis of novel silicon absorption gene from roots of mangrove (*Rhizophora apiculata*) *via* suppression subtractive hybridization BioMed Research International, Article ID 971985, 11 pages; 2014

Mahbod Sahebi, Mohamed M Hanafi, Siti Nor Akmal Abdullah, Mohd Y. Rafii, Parisa Azizi, F.F. Tengoua, A.B. Nurul Mayzaitul Azwa, M. Shabani mofrad. Silicon importance and mechanisms of bio-silica formation in plants. Submitted to BioMed Research International, 365704

Mahbod Sahebi, Mohamed M Hanafi, Siti Nor Akmal Abdullah, Mohd Y. Rafii, ParisaAzizi, Abu Seman Idris. *Serine-rich protein* is a novel positive regulator for silicon accumulation in mangrove plants. Submitted to GeneJournal.